Dephosphorylation Pathway of D-myo-Inositol 1,4,5-trisphosphate in the Unicellular Green Alga Chlamydomonas eugametos

Klerk, Hans; Himbergen, John A.J. van; Musgrave, Alan; Haastert, Peter J.M. van; Ende, Herman van den

Published in:
Phytochemistry

DOI:
10.1016/S0031-9422(00)94762-9

Publication date:
1994

Pergamon

Phytochemistry, Vol. 35, No. 2, pp. 349–352, 1994

0031 9422/94 $6.00 + 0.00

DEPHOSPHORYLATION PATHWAY OF D-*MYO*-INOSITOL 1,4,5-TRISPHOSPHATE IN THE UNICELLULAR GREEN ALGA *CHLAMYDOMONAS EUGAMETOS*

HANS KLERK,* JOHN A. J. VAN HIMBERGEN, ALAN MUSGRAVE, PETER J. M. VAN HAASTERT† and HERMAN VAN DEN ENDE‡

Institute for Molecular Cell Biology, BioCentrum Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands; †Department of Biochemistry, University of Groningen, Nijenborg 16, 9747 AG Groningen, The Netherlands

(*Received* 11 *June* 1993)

Key Word Index—*Chlamydomonas eugametos*; Chlorophyceae; inositol phosphate; dephosphorylation; signal transduction.

Abstract—*In vitro* dephosphorylation of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] by vegetative cells, gametes and zygotes of the green alga *Chlamydomonas eugametos* was studied using a soluble cell fraction as enzyme source and labelled Ins(1,4,5)P_3 as substrate. This compound was dephosphorylated yielding predominantly Ins(1,4)P_2, but in most cell types Ins(4,5)P_2 could also be detected. Both products were subsequently dephosphorylated to Ins(4)P, Ins(1)P and finally inositol. The study demonstrates that in these algae Ins(1,4,5)P_3 is degraded via a pathway that is characteristic of vertebrate rather than higher plant cells.

INTRODUCTION

It has been established in both animal and plant systems that D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] is an important second messenger for receptor-mediated Ca^{2+} mobilization [1, 2]. The central role of this compound implies that its metabolism is well regulated. From previous work it appears that Ins(1,4,5)P_3 degradation might be dependent on the type and developmental stage of the cells studied. In vertebrate cells, it is dephosphorylated by a 5-phosphatase to the biologically inactive Ins(1,4)P_2. This product is then further dephosphorylated yielding Ins(4)P and eventually inositol. Inositol is recycled into phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], the precursor of Ins(1,4,5)P_3. In this way, a cyclic metabolic pathway is established that is characteristic for this signalling system. In plant cells, on the other hand, Ins(1,4,5)P_3 degradation seems to occur mainly by a 1-phosphatase, yielding Ins(4,5)P_2 [3]. In *Dictyostelium discoideum*, both enzymes are operative, but their relative activity is dependent on the developmental stage. At the onset of development, both phosphatases are present in equal amounts, but during development the 5-phosphatase disappears [4, 5]. This raises the question whether specificity and developmental regulation of Ins(1,4,5)P_3 degradation can be demonstrated in other cell types. We have chosen the unicellular green alga *Chlamydomonas eugametos*, in which the Ins(1,4,5)P_3/Ca^{2+} signalling system has been shown to be present. *Chlamydomonas eugametos* proliferates vegetatively by binary fission, but under nitrogen starvation the cells undergo gametogenesis and become sexually competent. Gametes of opposite mating types (mt^+ and mt^-) agglutinate together via their flagellar surfaces. Receptor–receptor interaction triggers the formation of an intracellular signal that induces a mating structure to protrude through the anterior cell wall of each gamete [6]. Pairs of mt^+ and mt^- gametes eventually fuse together via their mating structures. There is evidence for the release of intracellular calcium during mating in *C. reinhardtii* [7, 8], suggesting a role for Ins(1,4,5)P_3. More recently, relative large quantities of Ptd(4,5)P_2 have been demonstrated in *C. eugametos*, with a relatively rapid turnover, as might be expected of a signal precursor [9–11]. It has also been shown that Ins(1,4,5)P_3 can induce mating structure activation in such gametes [12]. Thus, it is reasonable to believe that Ins(1,4,5)P_3 plays a role as second messenger in this species. In this study we have studied the degradation route of Ins(1,4,5)P_3 in extracts of gametes, zygotes and vegetative cells. It complements earlier work on the conversion of Ins(1,4,5)P_3 into higher phosphates [13].

RESULTS AND DISCUSSION

Dephosphorylation of Ins(1,4,5)P_3 in extracts of mt^+ gametes

When [2-^{3}H]Ins(1,4,5)P_3 was incubated with an extract of mt^+ gametes, three major products were formed:

*Present address: Department of Marine Biology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.
‡Author to whom correspondence should be addressed.

$InsP_2$, InsP and Ins (Fig. 1). This indicates that all the enzymes for $Ins(1,4,5)P_3$ degradation were present. A small decrease (*ca* 10%) in activity was found when Triton-X100 was omitted from the extraction buffer (not shown), suggesting that (part of) the enzymatic activity was membrane-associated. In non-dialysed extracts only 10% of the $Ins(1,4,5)P_3$ was degraded in 30 min and only 10% of the degraded products appeared as inositol. Dialysing the extract increased $Ins(1,4,5)P_3$ degradation to *ca* 65%, suggesting the presence of a phosphatase inhibitor of low molecular mass. Boiling the preparation did not destroy the inhibitor. It might be an inositol phosphate that was present in sufficient amount to compete with the radioactive substrates.

When intact living cells were incubated with [2-^{3}H]$Ins(1,4,5)P_3$ for 20 hr, *ca* 5% of the substrate was degraded into products that co-eluted with $InsP_2$, $InsP_1$ and Ins. However, this was also found when the radioactive substrate was incubated without cells. Further analysis of the degradation products proved that the majority of them could not be degraded by phytase and were therefore considered artifacts. We therefore conclude that the phosphatase activities in the cellular extracts are of intracellular origin.

To determine which phosphate group was removed first from $Ins(1,4,5)P_3$, a mixture of [4,5-^{32}P]$Ins(1,4,5)P_3$ (with 12% of the ^{32}P label at the 4-position [4]) and [2-^{3}H]$Ins(1,4,5)P_3$ was incubated with a gamete extract. In Fig. 2, the HPLC elution pattern of the products (B) is illustrated in comparison with a mixture of markers (A). A relatively large amount of ^{3}H and a small amount of ^{32}P co-eluted with $Ins(1,4)P_2$ and not with $Ins(4,5)P_2$. This indicates that most of the 5-phosphate is hydrolysed first, yielding $Ins(1,4)P_2$. The presence of ^{32}P label in this product is explained by its presence at the 4-position in the parent compound. However, this result does not exclude the possibility that some $Ins(4,5)P_2$ was also formed but was rapidly degraded, preventing its detection.

The parent compound [2-^{3}H]$Ins(1,4,5)P_3$ was also used to characterize the second dephosphorylation product, assuming that $Ins(1,4)P_2$ is a major intermediate. The InsP product eluted after the Ins(1)P standard at the position of Ins(4)P (Fig. 3). Thus, Ins(4)P was identified as the second intermediate in the degradation of $Ins(1,4,5)P_3$ to Ins.

Metabolism of Ins(1,4,5)P$_3$ in other cell types

The possibility, mentioned above, that $Ins(4,5)P_2$ is an intermediate in $Ins(1,4,5)P_3$ degradation was reinforced by the fact that it could readily be detected in assays using

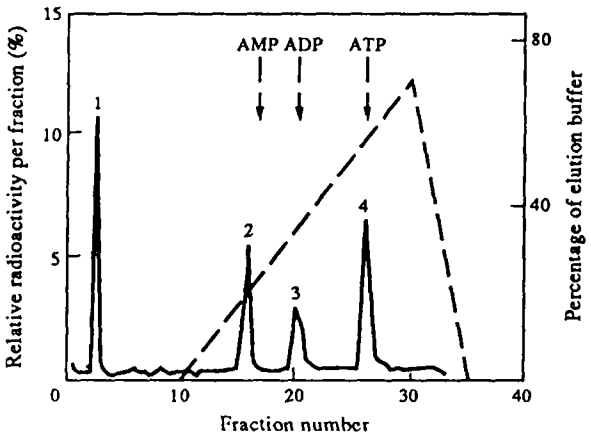

Fig. 1. HPLC elution pattern of [2-^{3}H]$Ins(1,4,5)P_3$ and its degradation products $InsP_2$, $InsP_1$ and Ins. [2-^{3}H]$Ins(1,4,5)P_3$ was incubated with *C. eugametos* extract for 30 min. The reaction products were separated on a Sorbax SAX column by elution with linear gradients formed from H_2O (X) and NH_4-Pi buffer, pH 3.7 (Y): 0 min, 0% Y; 10 min, 0% Y; 30 min, 70% Y; 35 min, 100% Y (dotted line). The flow rate was constant at 1 ml min^{-1}. Fractions were collected every 0.5 min and radioactivity in each fraction was determined by liquid-scintillation counting. The numbered peaks are 1, inositol; 2, $InsP_1$; 3, $InsP_2$; 4, $InsP_3$. Each peak was separately analysed by using shallow gradients. This allowed the different isomers to be identified.

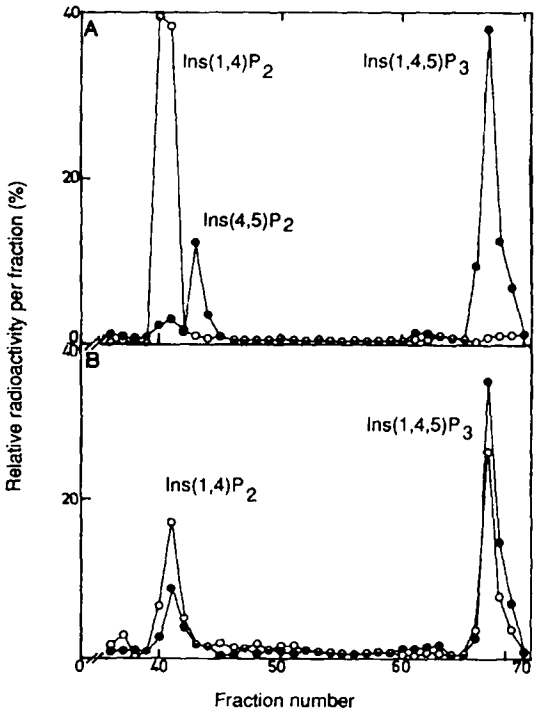

Fig. 2. Analysis of the first degradation product of $Ins(1,4,5)P_3$ after incubation with *C. eugametos* gamete mt$^+$ extract. A mixture of [5-^{32}P]$Ins(1,4,5)P_3$, [5-^{32}P]$Ins(4,5)P_2$ and [^{3}H]$Ins(1,4)P_2$ were separated as markers and a mixture of [2-^{3}H]$Ins(1,4,5)P_3$ and [5-^{32}P]$Ins(1,4,5)P_3$ was incubated with extract for 30 min. The markers (A) and degradation products (B) were separated and detected as described in Fig. 1. The column was eluted with linear gradients formed from H_2O (X) and NH_4-Pi buffer, pH 3.7 (Y) to separate the different isomers of $InsP_2$: 0 min, 0% Y; 10 min, 0% Y; 11 min, 8% Y; 23 min, 20% Y; 28 min, 100% Y; 38 min, 100% Y. The flow rate was 1 ml min^{-1} and fractions of 0.5 ml were collected. Closed symbols, [^{32}P] label; open symbols, [^{3}H] label.

other cell types. Extracts were prepared from zygotes as well as from vegetative cells and gametes of both strains of *C. eugametos* and incubated with Ins(1,4,5)P_3. The phosphatase activity was highest in extracts of gametes and vegetative mt^+ cells and lowest in the vegetative mt^- cells and zygotes (Table 1). When the latter two extracts were mixed with that of mt^+ gametes, intermediate levels of activity were found, indicating that low activities were not due to inhibitors in the extracts (not shown). The first degradation product was mainly Ins(1,4)P_2, but Ins(4,5)P_2 was clearly present, except in extracts of mt^+ gametes, as shown above. It is apparent that there is an inverse correlation between the contribution of 1-phosphatase activity and the overall Ins(1,4,5)P_3 degradation activity (Table 1). This suggests that a low level of 1-phosphatase activity is present in all cell types, but is masked in those cells with high 5-phosphatase activity, such as mt^+ gametes.

The second degradation product in the cell extracts was mainly Ins(4)P. In mt^- vegetative cells the high proportion of Ins(1)P was remarkable. This suggests the presence of a very active 4-phosphatase, which as far as we know, has not been described previously. In all extracts Ins(1,4,5)P_3 was finally degraded to inositol (not shown).

Taken together, these results indicate that in *C. eugametos*, the dephosphorylation of Ins(1,4,5)P_3 is of a mixed type, with a 1-, 4- and 5-phosphatase being involved, dependent on the strain. Generally, the action of the 5-phosphatase was predominant, corresponding to the dephosphorylation pattern found in vertebrates. This is in agreement with the fact that the route by which higher inositol phosphates are synthesized in extracts of *C. eugametos* gametes also resembles that of higher animals rather than *Dictyostelium* or plants [13]. Differences between the various developmental stages were apparent, suggesting that the dephosphorylation pattern is modulated, as found in *Dictyostelium*. This may result in changes in the steady state level of Ins(4,5)P_2 and Ins(1,4)P_2 that may be significant in analogy with erythrocytes, where Ins(4,5)P_2 as opposed to Ins(1,4)P_2 has been shown to have signal properties [14]. Thus, by favouring a particular degradation pathway, the level of biologically active intermediates can be modulated.

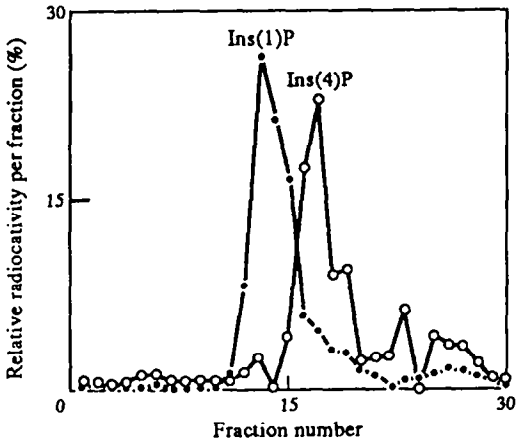

Fig. 3. Analysis of the second (InsP) products. The HPLC conditions were as described in Fig. 1. [^{3}H]Ins(4)P and [^{14}C]Ins(1)P were used as markers. To separate the isomers the column was eluted with linear gradients formed from H_2O (X) and NH_4-Pi buffer, pH 3.7 (Y): 0 min, 0% Y; 10 min, 0% Y; 11 min, 8% Y; 23 min, 20% Y; 28 min, 100% Y; 38 min, 100% Y. The flow rate was 2 ml min^{-1} and fractions of 0.1 ml were collected. Closed symbols, [^{14}C] label; open symbols, [^{3}H] label.

EXPERIMENTAL

Materials. [2-^{3}H]Ins(1,4,5)P_3, [5-^{32}P]Ins(1,4,5)P_3 (with 12% of the label at the 4-position) and [^{3}H]Ins(4)P were from New England Nuclear (Den Bosch, The Netherlands); [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(4)P and [^{14}C]Ins(1)P were from Amersham Int. [^{32}P]Ins(4,5)P_2 was prepd from [5-^{32}P]Ins(1,4,5)P_3 using 1-phosphatase isolated from *D. discoideum* [4]. Phenylmethylsulphonyl fluoride (PMSF), aprotenin, leupeptin, dithiothreitol, Dowex-1 and phytase were from Sigma.

Cell cultures. Chlamydomonas eugametos strains 17.17.2 (mt^+) and 5.39.4 (mt^-) were grown in Petri dishes on agar-containing medium [15]. Gamete suspensions were obtained by flooding 2- to 4-week-old cultures with H_2O. Vegetative cells of the same strains were produced by growing them for 4 days in 500 ml of liquid medium. The cells were tested for the presence of gametes by mixing them with gametes of opposite mating type. The absence of agglutination indicated that the cells were vegetative. Primary zygotes were produced by mixing mt^+ and mt^- gamete suspensions in equal amounts in 15-cm Petri

Table 1. Dephosphorylation of [2-^{3}H]Ins(1,4,5)P_3 in extracts of different cell types of *C. eugametos* and the relative abundance of the InsP_1 and InsP_2 isomers recovered

Cell type	Per cent of InsP_3 degraded (μg^{-1} protein min^{-1})	Ratio of degradation products Ins(1,4)P_2 : Ins(4,5)P_2	Ins(1)P : Ins(4)P
mt^+ Gametes	1.8	100:0	0:100
mt^- Gametes	1.6	84:16	31:69
mt^+ Veg. cells	1.5	90:10	33:67
mt^- Veg. cells	0.5	71:29	50:50
Zygotes	0.2	64:36	0:100

dishes. After 4–6 hr, vis-a-vis pairs began to aggregate in large patches on the bottom of each dish. After 18 hr, free-swimming gametes and agglutinating gametes were removed by suction in a pipette. Developing zygotes remained adhered to the bottom of the Petri dish. They were washed twice with H_2O and then brought into suspension by scraping them off with a piece of plastic tubing. *Ca* 3 ml of a dense suspension of these cells (*ca* 10^7 ml^{-1}) was then inoculated on to agar medium in a Petri dish. After drying the agar surface in a stream of sterile air, the culture was left for 24 hr at 20° under continuous light (100 μmol photons m^{-1} sec^{-1}). The pairs had then developed into round or sausage-shaped primary zygotes.

Enzyme extracts. *Ca* 2×10^9 cells were centrifuged for 5 min at 1000 g. The pellet was washed with 10 vol of ice-cold buffer (20 mM HEPES–NaOH, 0.5 mM EDTA, pH 7), and resuspended in 5 vol of extraction buffer (20 mM HEPES–NaOH, 0.5 mM PMSF, 1 μg ml^{-1} aprotenin, 1 μg ml^{-1} leupeptin, 1 mM dithiothreitol, 1% Triton X-100, pH 7). Cells were then disrupted by sonication and the resulting homogenate was centrifuged for 10 min at 10 000 g. The supernatant was recentrifuged for 10 min at 150 000 g and the clear extract was dialysed twice for 6 and 16 hr against 100 vol of extraction buffer. During the whole extraction procedure the temp. did not exceed 4°.

Phosphatase assay. The dephosphorylation of inositol phosphates was assayed in extraction buffer in the presence of 5 mM $MgCl_2$ at 20°. Incubation was started by adding 10 μl of the *Chlamydomonas* extract to 10 μl assay mixt. containing 60 000–180 000 Bq radioactive substrate. The reaction was stopped after 10–60 min by boiling for 5 min (for HPLC analysis) or by adding 0.5 ml $CHCl_3$–MeOH–35% HCl (20:40:1) prior to chromatography on Dowex-1.

HPLC analysis. Labelled inositol phosphates and their isomers were sepd by HPLC on a Sorbax SAX column (Du Pont, Wilmington, U.S.A.) using a gradient system with increasing conc of a NH_4-Pi buffer, pH 3.7, in H_2O. The column was equilibrated with H_2O before each run. Aliquots (5 μl) of 5 mM AMP, ADP and ATP were included as int. standards in each sample and their elution was monitored by *A* at 259 nm. Eluant samples of 0.25, 0.50 or 1 ml were collected and the radioactivity determined after adding 2 ml scintillation fluid (Emulsifier 299, Packard).

Dowex anion exchange chromatography. The method was essentially as described earlier [4]. Phosphatase assays were stopped with $CHCl_3$–MeOH–35% HCl (20:40:1). To each sample 200 μl of H_2O was added, the mixt. shaken vigorously and the phases sepd by centrifugation (1 min, 10 000 g). The aq. phase was applied to a 0.5 ml Dowex-1 anion exchange column (formate form). The different reaction products were sepd by stepwise elution with: (i) 10 ml of H_2O (Ins); (ii) 10 ml of 150 mM NH_4-formate–5 mM $Na_2B_4O_7$ (InsP and Pi); (iii) 10 ml 300 mM NH_4-formate–100 mM formic acid ($InsP_2$), and (iv) 10 ml 750 mM NH_4-formate–100 mM formic acid ($InsP_3$). Radioactivity in the frs was measured after adding 10 ml of scintillation fluid.

REFERENCES

1. Berridge, M. J. and Irvine, R. F. (1989) *Nature* **241**, 197.
2. Trewavas, A. and Gilroy, S. (1991) *Trends in Genetics* 7, 356.
3. Drobak, B. K., Watkins, P. A. C., Chattaway, J. A., Roberts, K. and Dawson, A. P. (1991) *Plant Physiol.* **95**, 412.
4. Van Lookeren Campagne, M. M., Erneux, C., Van Eijk, R. V. and Van Haastert, P. J. M. (1988) *Biochem. J.* **254**, 343.
5. Bominaar, A. A., Van Dijken, P., Drayer, R. and Van Haastert, P. J. M. (1991) *Differentiation* **46**, 1.
6. Van den Ende, H., Musgrave, A. and Klis, F. M. (1990) in *Ciliary and Flagellar Membranes* (Bloodgood, R. A., ed.), pp. 129–147. Plenum Press, NY.
7. Bloodgood, R. A. and Levin, E. N. (1983) *J. Cell Biol.* **97**, 397.
8. Kaska, D. D., Piscopo, I. C. and Gibor, A. (1985) *Exp. Cell Res.* **160**, 371.
9. Irvine, R. E., Letcher, A. J., Lander, D. L., Drobak, B. K., Dawson, A. P. and Musgrave, A. (1989) *Plant Physiol.* **89**, 888.
10. Brederoo, J., De Wildt, P., Popp-Snijders, C., Irvine, R. F., Musgrave, A. and Van den Ende, H. (1991) *Planta* **184**, 175.
11. Musgrave, A., Kuin, H., Jongen, M., De Wildt, P., Schuring, F., Klerk, H. and Van den Ende, H. (1992) *Planta* **186**, 442.
12. Schuring, F., Brederoo, J., Musgrave, A. and Van den Ende, H. (1990) *FEMS Microbiol. Letters* **71**, 237.
13. Irvine, R. F., Letcher, A. J., Stephans, L. R. and Musgrave, A. (1992) *Biochem. J.* **281**, 361.
14. Mason-Garcia, M. and Beckman, B. S. (1991) *FASEB J.* **5**, 2958.
15. Schuring, F., Smeenk, J. W., Homan, W. L., Musgrave, A. and Van den Ende, H. (1987) *Planta* **170**, 322.